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(57) Abstract

The present invention is directed to a method of producing a cereal plant cell useful for regeneration to a low temperature stress, water stress, or salt stress tolerant cereal plant by transforming the cereal plant cell with a nucleic acid encoding a cold regulated protein. In addition, a method of increasing tolerance of a cereal plant to low temperature stress, water stress, or salt stress is also provided. A transgenic cereal plant or cereal plant cell transformed with a nucleic acid encoding a cold regulated protein is also provided.

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PRODUCTION OF LOW-TEMPERATURE, SALT- AND DROUGHT-TOLERANT TRANSGENIC CEREAL PLANTS

FIELD OF THE INVENTION

The present invention relates generally to transgenic cereal plants, and more particularly to transgenic cereal plants which comprise nucleic acid encoding a cold-regulated protein which confers low-temperature stress, water stress, or salt stress tolerance on the transgenic cereal plants.

BACKGROUND OF THE INVENTION

Throughout this application various publications are referenced, many in parenthesis. Full citations for these publications are provided at the end of the Detailed Description. The disclosures of these publications and referenced U.S. Patents and U.S. Patent applications in their entireties are hereby incorporated by reference in this application.

Environmental stresses, such as drought, increased salinity of soil, and extreme temperature, are major factors in limiting plant growth and productivity. The worldwide loss in yield of three major cereal crops, rice, maize (corn), and wheat due to water stress (drought) has been estimated to be over ten billion dollars annually. Breeding of stress-tolerant crop cultivars represents a promising strategy to tackle these problems (Epstein et al., 1980). However, conventional breeding is a slow process for generating crop varieties with improved tolerance to stress conditions. Limited germplasm resources for stress tolerance and incompatibility in crosses between distantly related plant species are additional problems encountered in conventional breeding. Recent progress in plant genetic transformation and availability of potentially useful genes characterized from different sources make it possible to generate salt or drought stress-tolerant crops using transgenic approaches (Tarczynski et al., 1993; Pilon-Smits et al., 1995).

Characterization and cloning of plant genes that confer stress tolerance remains a challenge. Genetic studies revealed that tolerance to drought and salinity in some crop varieties is principally due to additive gene effects (Akbar et al., 1986a,

1986b). However, the underlying molecular mechanism for the tolerance has never been revealed. Physiological and biochemical responses to high levels of ionic or nonionic solutes and decreased water potential have been studied in a variety of plants. Based on accumulated experimental observations and theoretical consideration, one suggested mechanism that may underlie the adaptation or tolerance of plants to osmotic stresses is the accumulation of compatible, low molecular weight osmolytes such as sugar alcohols, special amino acids, and glycinebetaine (Greenway and Munns, 1980; Yancey et al., 1982). Recently, a transgenic study has demonstrated that accumulation of the sugar alcohol mannitol in transgenic tobacco conferred protection against salt stress (Tarczynski et al., 1993). Two recent studies using a transgenic approach have demonstrated that metabolic engineering of the glycinebetaine biosynthesis pathway is not only possible but also may eventually lead to production of stress-tolerant plants (Holmstrom et al., 1994; Rathinasabapathi et al., 1994).

In addition to metabolic changes and accumulation of low molecular weight compounds, a large set of genes is transcriptionally activated which leads to accumulation of new proteins in vegetative tissue of plants under osmotic stress conditions (Skriver and Mundy, 1990; Chandler and Robertson, 1994). The expression levels of a number of genes have been reported to be correlated with desiccation, salt, or cold tolerance of different plant varieties of the same species. A number of proteins have been reported to accumulate in plants in response to salinity stress or drought stress (water deficit). The stress-induced proteins include the late embryogenesis abundant ("LEA") family (Xu, et al., 1996), dehydrines, COR47 (Gilmour, et al., 1992) and lti30 and lti45 (Welin, et al., 1994). Since a number of proteins and osmolytes are produced under abiotic stress, it is not known which of them, if any, may be responsible for protecting the plants against these stresses. Further, the functions of many stress-responsive genes are unknown. Elucidating the function of these stress-responsive genes will not only advance understanding of plant adaptation and tolerance to environmental stresses, but also may provide important information for designing new strategies for crop improvement (Chandler and Robertson, 1994).

A need exists, therefore, for the identification of a gene encoding a protein that can confer low temperature stress, water stress, or salt stress tolerance on a plant transformed with the gene. Such a low temperature stress, water stress, or salt stress tolerant plant can find many uses, particularly in agriculture and particularly in regard to cereal plants, which are major crop plants.

SUMMARY OF INVENTION

The present invention relates to a method of producing a cereal plant cell useful for regeneration to a low temperature stress, water stress, or salt stress tolerant cereal plant by transforming a cereal plant cell with a nucleic acid encoding a cold regulated protein.

Another aspect of the present invention relates a cereal plant cell transformed with a nucleic acid encoding a cold regulated protein that confers low temperature stress, water stress, or salt stress tolerance on a cereal plant regenerated from the cereal plant cell.

Yet another aspect of the present invention relates to a seed produced by the transgenic cereal plants according to the subject invention, and seed which, upon germination, produces the transgenic cereal plants of the subject invention.

The invention additionally provides a method of increasing tolerance of a cereal plant to low temperature stress, water stress, or salt stress conditions. The method includes increasing levels of a cold regulated protein in the cereal plant. This can be accomplished by introducing a promoter and a nucleic acid encoding a cold regulated protein and transforming the cereal plant.

By introducing nucleic acid molecules of the present invention into a transgenic cereal plant, transgenic cereal plants having significantly increased tolerance to low temperature, water stress (drought), and salt stress are produced. Thus, these genes can be used as molecular tools for genetic crop improvement by conferring stress tolerance.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features and advantages of this invention will be evident from the following detailed description of preferred embodiments when read in conjunction with the accompanying drawing in which:

Figure 1 shows the structure of the plasmid pCU1a for expression of *COR47* in transgenic rice. Only common restriction endonuclease sites are indicated. The DNA fragment used as a probe in DNA blot hybridization is also indicated.

Figure 2 shows the structure of the plasmid pBY520 for expression of *HVA1* in transgenic rice. Only common restriction endonuclease sites are indicated. The DNA fragment used as a probe in DNA blot hybridization is also indicated.

DETAILED DESCRIPTION

The invention provides a method of producing a cereal plant cell useful for regeneration to a low temperature stress, water stress, or salt stress tolerant cereal plant by transforming a cereal plant cell with a nucleic acid encoding a cold regulated protein. Once transformation has occurred, the cereal plant cell can be regenerated to form a transgenic cereal plant.

The invention is also directed to a method of increasing tolerance of a cereal plant to low temperature stress, water stress, or salt stress conditions. The method comprises increasing levels of a cold regulated protein in the cereal plant. This can be accomplished by controlling expression of a heterologous cold regulated protein gene with a strong promoter in the cereal plant.

Cereal which can be transformed in accordance with the subject invention are members of the family *Gramineae* (also known as *Poaceae*), and include rice (genus *Oryza*), wheat, corn, barley, oat, sorghum, and millet. Preferably, the cereal is rice, wheat, or corn, and most preferably the cereal is rice. Many species of cereals can be transformed, and within each species the numerous subspecies and varieties can be transformed. For example, within the rice species is subspecies Indica rice (*Oryza sativa* ssp. Indica), which includes the varieties IR36, IR64, IR72, Pokkali, Nona Bokra, KDML105, Suponburi 60, Suponburi 90, Basmati 385, and Pusa Basmati 1. Another rice subspecies is Japonica, which includes Nipponbere,

Kenfeng, and Tainung 67. Examples of suitable maize varieties include A188, B73, VA22, L6, L9, K1, 509, 5922, 482, HNP, and IGES. Examples of suitable wheat varieties include Pavon, Anza, Chris, Coker 983, FLA301, FLA302, Fremont, and Hunter.

Having identified the cereal plant of interest, plant cells suitable for transformation include immature embryos, calli, suspension cells, and protoplasts.

These cereal plant cells are transformed with a nucleic acid, which could be RNA or DNA and which is preferably cDNA, encoding a cold regulated protein. The nucleic acid can be biologically isolated or synthetic. Preferably, the cold regulated proteins are COR47, COR6.6, LTI30, or LTI45 proteins. In particular, the COR47 protein is encoded by the *COR47* gene of *Arabidopsis thaliana* L. having the nucleic acid sequence and amino acid sequence identified in Gilmour et al., 1992, the COR6.6 protein is encoded by the *COR6.6* gene of *Arabidopsis thaliana* L. having the nucleic acid sequence and amino acid sequence identified in Gilmour et al., 1992, the LTI30 protein is encoded by the *LTI30* gene of *Arabidopsis thaliana* L. having the nucleic acid sequence and amino acid sequence as disclosed in Welin et al., 1994, and the LTI45 protein is encoded by the *LTI45* gene of *Arabidopsis thaliana* L. having the nucleic acid sequence and amino acid sequence as disclosed in Welin et al., 1994.

The DNA molecule encoding the cold regulated protein of the present invention can be incorporated in cells using conventional recombinant DNA technology. Generally, this involves inserting the DNA molecule into an expression system to which the DNA molecule is heterologous (i.e. not normally present). The heterologous DNA molecule is inserted into the expression system or vector in proper sense orientation and correct reading frame. The vector contains the necessary elements for the transcription and translation of the inserted protein-coding sequences. Alternatively, the heterologous DNA molecule of the present invention encoding the cold regulated protein can impart resistance to plants containing that molecule by the RNA-mediated resistance mechanism.

Once the isolated DNA molecule encoding the cold regulated protein, as described above, has been cloned into an expression system, it is ready to be incorporated into a host cell. Such incorporation can be carried out by the

various forms of transformation noted above, depending upon the vector/host cell system. Suitable host cells include, but are not limited to, bacteria, virus, yeast, mammalian cells, insect, plant, and the like.

The present invention also relates to RNA molecules which encode the cold regulated protein. The transcripts can be synthesized using the host cells of the present invention by any of the conventional techniques.

Preferably, transformation of plant cells is accomplished by using a plasmid. The plasmid is used to introduce the nucleic acid encoding the cold regulated protein into the plant cell. Accordingly, a plasmid preferably includes DNA encoding the cold regulated protein inserted into a unique restriction endonuclease cleavage site. DNA is inserted into the vector using standard cloning procedures readily known in the art. This generally involves the use of restriction enzymes and DNA ligases, as described by Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2d edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989). The resulting plasmid which includes a nucleic acid encoding an cold regulated protein can then be used to transform a host cell, such as a plant cell.

For plant transformation, the plasmid preferably also includes a selectable marker for plant transformation. Commonly used plant selectable markers include the hygromycin phosphotransferase (*hpt*) gene, the phosphinothricin acetyl transferase gene (*bar*), the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), neomycin 3'-O-phosphotransferase (*npt* II), or acetolactate synthase (ALS).

The plasmid preferably also includes suitable promoters for expression of the nucleic acid encoding the cold regulated protein and for expression of the marker gene. The rice actin 1 gene promoter, the maize ubiquitin promoter, and the cauliflower mosaic virus 35S promoter are commonly used for plant transformation. In plasmid pCU1a which is used in the following examples, the nucleic acid encoding the cold regulated protein is under the control of the constitutive rice actin 1 gene promoter and the marker gene (*bar*) is under control of the cauliflower mosaic virus 35S promoter. Other promoters useful for plant transformation with the cold regulated gene include those from the genes encoding ubiquitin, proteinase inhibitor II (PINII), and rice actin 4, as well as stress-induced promoters (such as the HVA1 gene promoter of barley) and cold inducible promoter (Welin et al., 1994).

The plasmid designated pCU1a has been deposited as DNA pursuant to, and in satisfaction of, the requirements of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure, with the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, Virginia 20110-2209 under ATCC Accession No. _____ on June 26, 1998.

For plant transformation, the plasmid also preferably includes a nucleic acid molecule encoding a 3' terminator such as that from the 3' non-coding region of genes encoding a proteinase inhibitor, actin, or nopaline synthase (nos).

Other suitable plasmids for use in the subject invention can be constructed. For example, cold regulated genes other than the cold regulated genes of *Arabidopsis thaliana* L. could be ligated into plasmid pCU1a after use of restriction enzymes to remove the cold regulated gene. Other promoters could replace the actin 1 gene promoter present in pCU1a. Alternatively, other plasmids in general containing cold regulated genes under the control of a suitable promoter, with suitable selectable markers, can be readily constructed using techniques well known in the art.

Having identified the plasmid, one technique of transforming cereal plant cells with a gene which encodes for an cold regulated protein is by contacting the plant cell with an inoculum of a bacteria transformed with the plasmid comprising the gene that encodes for the cold regulated protein. Generally, this procedure involves inoculating the plant cells with a suspension of the transformed bacteria and incubating the cells for 48 to 72 hours on regeneration medium without antibiotics at 25-28°C.

Bacteria from the genus *Agrobacterium* can be utilized to transform plant cells. Suitable species include *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*. *Agrobacterium tumefaciens* (e.g., strains LBA4404 or EHA105) is particularly useful due to its well-known ability to transform plants (Hiei, et al., 1994).

In inoculating the cells of cereal plants with *Agrobacterium* according to the subject invention, the bacteria must be transformed with a plasmid which includes a gene encoding for an cold regulated protein.

One method for introduction of a plasmid containing nucleic acid encoding a cold regulated protein into a plant cell is by transformation of the plant cell nucleus, such as by particle bombardment. As used throughout this application, particle bombardment (also know as biolistic transformation) of the host cell can be accomplished in one of several ways. The first involves propelling inert or biologically active particles at cells. This technique is disclosed in U.S. Patent Nos. 4,945,050, 5,036,006, and 5,100,792, all to Sanford et al., which are hereby incorporated by reference. Generally, this procedure involves propelling inert or biologically active particles at the cells under conditions effective to penetrate the outer surface of the cell and to be incorporated within the interior thereof. When inert particles are utilized, the plasmid can be introduced into the cell by coating the particles with the plasmid containing the heterologous DNA. Alternatively, the target cell can be surrounded by the plasmid so that the plasmid is carried into the cell by the wake of the particle. Biologically active particles (e.g., dried bacterial cells containing the plasmid and heterologous DNA) can also be propelled into plant cells.

A further method for introduction of the plasmid into a plant cell is by transformation of plant cell protoplasts (stable or transient). Plant protoplasts are enclosed only by a plasma membrane and will therefore take up macromolecules like heterologous DNA. These engineered protoplasts can be capable of regenerating whole plants. Suitable methods for introducing heterologous DNA into plant cell protoplasts include electroporation and polyethylene glycol (PEG) transformation. As used throughout this application, electroporation is a transformation method in which, generally, a high concentration of plasmid DNA (containing heterologous DNA) is added to a suspension of host cell protoplasts and the mixture shocked with an electrical field of 200 to 600 V/cm. Following electroporation, transformed cells are identified by growth on appropriate medium containing a selective agent.

As used throughout this application, transformation encompasses stable transformation in which the plasmid is integrated into the plant chromosomes.

In the Examples which follow, rice has been transformed using biolistic transformation. Other methods of transformation have also been used to successfully transform rice plants, including the protoplast method (for a review, see Cao et al., 1992), and the *Agrobacterium* method (Hiei et al., 1994). Biolistic

transformation has also been used to successfully transform maize (for a review, see Mackey et al., 1993) and wheat (see U.S. Patent No. 5,405,765 to Vasil et al.).

Once a cereal plant cell or protoplast is transformed in accordance with the present invention, it is regenerated to form a transgenic cereal plant. Generally, regeneration is accomplished by culturing transformed cells or protoplasts on medium containing the appropriate growth regulators and nutrients to allow for the initiation of shoot meristems. Appropriate antibiotics are added to the regeneration medium to inhibit the growth of *Agrobacterium* or other contaminants and to select for the development of transformed cells or protoplasts. Following shoot initiation, shoots are allowed to develop in tissue culture and are screened for marker gene activity.

In suitable transformation methods, the cereal plant cell to be transformed can be *in vitro* or *in vivo*, i.e. the cereal plant cell can be located in a cereal plant.

In addition, the method of the present invention may include transforming the cereal plants with a second nucleic acid, which could be RNA or DNA, and which is preferably, cDNA, encoding a cold regulated protein or a late embryogenesis abundant ("LEA") protein. Transformation with a second nucleic acid encoding a cold regulated protein will increase the tolerance of the transformed cereal plant to salt, drought, and low temperature stress. Transformation with a nucleic acid encoding a LEA protein will increase the tolerance of the transformed cereal plant to salt and drought stress. Preferably, the second nucleic acid encodes a LEA protein and transformation is accomplished using any of the techniques described above including using the plasmid designated pBY520 as described in Xu et al., 1996 and U.S. Patent Application No. 08/730,659 to Wu. The plasmid designated pBY520 has been deposited in *Escherichia coli* strain pBY520/DH5α pursuant to, and in satisfaction of, the requirements of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure, with the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, Virginia 20110-2209 under ATCC Accession No. 69930 on October 12, 1995.

In the following Examples, the LEA protein is encoded by the *HVA1* gene of barley, having the nucleotide and amino acid sequences as disclosed in Straub

et al. (1994). However, other LEA genes can also be utilized, particularly other LEA genes belonging to group 3. These other group 3 LEA genes include cotton D-7 and D-29 (Baker et al., 1988; Dure et al., 1981), *Brassica* pLEA76 (Harada et al., 1989), carrot Dc8 and Dc3 (Franz et al., 1989; Seffens et al., 1990), soybean pmGM2 (Hsing et al., 1992), and wheat pMA2005 and pMA1949 (Curry et al., 1991; Curry and Walker-Simmons, 1991). The published nucleotide and amino acid sequences of each of these LEA proteins are hereby incorporated by reference. Each of these sequences can be used as the nucleic acid encoding an LEA protein to transform a suitable cereal plant according to the subject invention. Other LEA genes of group 2 or group 1 can also be used. Various LEA genes are disclosed in Dure (1992).

The invention also provides a transgenic cereal plant produced by the method of the subject invention, as well as seed produced by the transgenic cereal plant.

The invention further provides a cereal plant cell or protoplast or a transgenic cereal plant transformed with a nucleic acid encoding a cold regulated protein that confers low temperature stress, water stress, or salt stress tolerance to the plant generated from the cereal plant cell or protoplast or to the transgenic cereal plant. As discussed above, various cereal plants and cold regulated genes can be utilized.

Preferably, the nucleic acid encoding an cold regulated protein is controlled by a strong promoter to effect maximum expression of the cold regulated protein, or by a stress-induced promoter to effect induction of the promoter in response to stress conditions. In one embodiment, the transgenic cereal plant cell or protoplast or plant is transformed with the nucleic acid encoding the promoter, such as the rice actin 1 gene promoter, by providing a plasmid which includes DNA encoding the cold regulated gene and the promoter.

The transgenic cereal plant cell or protoplast or plant can also be transformed with a nucleic acid encoding a selectable marker, such as the *bar* gene, to allow for detection of transformants, and with a nucleic acid encoding the cauliflower mosaic virus 35S promoter to control expression of the *bar* gene. Other selectable markers include genes encoding EPSPS, nptII, or ALS. Other promoters include those from genes encoding actin 1, ubiquitin, and PINII. These additional nucleic

acid sequences can also be provided by the plasmid containing the cold regulated gene and its promoter. Where appropriate, the various nucleic acids could also be provided by transformation with multiple plasmids.

The invention is also directed to a transgenic cereal plant regenerated from the transgenic cereal plant cells or protoplasts, as well as to seed produced by the transgenic cereal plants. The invention is also directed to seed, which upon germination, produces the transgenic cereal plant.

While the nucleotide sequence referred to herein encodes a cold regulated protein or a LEA protein, nucleotide identity to a previously sequenced cold regulated protein or the LEA protein is not required. As should be readily apparent to those skilled in the art, various nucleotide substitutions are possible which are silent mutations (i.e. the amino acid encoded by the particular codon does not change). It is also possible to substitute a nucleotide which alters the amino acid encoded by a particular codon, where the amino acid substituted is a conservative substitution (i.e. amino acid "homology" is conserved). It is also possible to have minor nucleotide and/or amino acid additions, deletions, and/or substitutions in the cold regulated or LEA protein nucleotide and/or amino acid sequences which have minimal influence on the properties, secondary structure, and hydrophilic/hydrophobic nature of the encoded protein. These variants are encompassed by the nucleic acid encoding an cold regulated or LEA protein according to the subject invention.

Also encompassed by the present invention are transgenic cereal plants transformed with fragments of the nucleic acids encoding the cold regulated proteins of the present invention. Suitable fragments capable of conferring low temperature stress, water stress, or salt stress tolerance to cereal plants can be constructed by using appropriate restriction sites. A fragment refers to a continuous portion of the cold regulated encoding molecule that is less than the entire molecule.

Non-essential nucleotides could be placed at the 5' and/or 3' end of the fragments (or the full length molecules) without affecting the functional properties of the fragment or molecule (i.e. in increasing low temperature stress, water stress, or salt stress tolerance). For example, the nucleotides encoding the protein may be conjugated to a signal (or leader) sequence at the N-terminal end (for example) of the protein which co-translationally or post-translationally directs transfer of the protein.

The nucleotide sequence may also be altered so that the encoded protein is conjugated to a linker or other sequence for ease of synthesis, purification, or identification of the protein.

EXAMPLES

Materials and Methods

Construction of *Act1-COR47* Plasmid for Rice Transformation

A 1.0-kb *Eco*RI fragment containing the full-length *COR47* cDNA was isolated from the cDNA clone (Gilmour et al., 1992), and this fragment was blunted with Klenow DNA polymerase and subcloned into the *Sma*I site of the plasmid expression vector pBY505, which is a derivative of pBluescriptIIKS(+)(Stratagene, CA), to create pCU1a. On pCU1a, the *COR47* structural gene is regulated by rice actin 1 gene (*Act1*) promoter (McElroy et al., 1990; Zhang, et al, 1991) and is between the *Act1* promoter and the potato proteinase inhibitor II gene (*Pin2*) 3' non-coding region (Thornburg et al., 1987). Plasmid pCU1a also contains the bacterial phosphinothricin acetyl transferase (PAT) structural gene (commonly known as *bar* gene) (White et al., 1990), which serves as the selectable marker in rice transformation by conferring resistance to phosphinothricin-based herbicides. The *bar* gene is regulated by the cauliflower mosaic virus (CaMV) 35S promoter and followed by the nopaline synthase gene (*nos*) termination signal. Plasmid pCU1a has been deposited with the ATCC under Accession No._____.

Construction of *Act1-HVA1* Plasmid for Rice Transformation

A 1.0-kb *Eco*RI fragment containing the full-length *HVA1* cDNA was isolated from the cDNA clone *pHVA1* (Hong et al., 1988), and this fragment was blunted with Klenow DNA polymerase and subcloned into the *Sma*I site of the plasmid expression vector pBY505, which is a derivative of pBluescriptIIKS(+)(Stratagene, CA), to create pBY520. On pBY520, the *HVA1* structural gene is regulated by rice actin 1 gene (*Act1*) promoter (McElroy et al., 1990; Zhang, et al, 1991) and is between the *Act1* promoter and the potato proteinase inhibitor II gene (*Pin2*) 3N region (Thornburg et al., 1987). Plasmid pBY520 also

contains the bacterial phosphinothricin acetyl transferase (PAT) structural gene (commonly known as *bar* gene) (White et al., 1990), which serves as the selectable marker in rice transformation by conferring resistance to phosphinothricin-based herbicides. The *bar* gene is regulated by the cauliflower mosaic virus (CaMV) 35S promoter and followed by the nopaline synthase gene (*nos*) termination signal. Plasmid pBY520 has been deposited with the ATCC under Accession No. 69930.

Production of Transgenic Rice Plants

Calli were induced from mature embryos of rice (*Oryza sativa* L c.v. Nipponbare; available from the International Rice Research Institute, Los Banos, Philippines) or suspension cultures established from selected embryogenic calli after three months of subculture in liquid medium. Fine suspension culture cells were used as the transformation material and bombarded with tungsten particles coated with the pCUla or pBY520 plasmid as described by Cao et al. (1992). Resistant calli were selected in selection medium containing 6 mg/l of ammonium glufosinate (Crescent Chemical Co., Hauppauge, NY) as the selective agent for 5-7 weeks. The resistant calli were transferred to MS (Murashige and Skoog, 1962) regeneration medium containing 3 mg/l of ammonium glufosinate to regenerate into plants. Plants regenerated from the same resistant callus were regarded as clones of the same line. Regenerated plants were transferred into soil and grown in the greenhouse (32°C day/22°C night, with a supplemental photoperiod of 10 h).

Herbicide-Resistance Test of Transgenic Rice Plants

The presence of the transferred genes in regenerated rice plants was first indicated by herbicide resistance of the plants. For the herbicide-resistance test, a water solution containing 0.5% (V/V) commercial herbicide BASTA™ (containing 162 g/l glufosinate ammonium, Hoechst-Roussel Agri-Vet Company, Somerville, NJ) and 0.1% (V/V) Tween-20 was painted on both sides of a leaf. After one week, the resistant/sensitive phenotype was scored. Treated leaves of nontransformed ("NT") plants were severely damaged or died, whereas the treated leaves of transgenic plants were not affected or only slightly damaged in the treated areas.

DNA Blot Hybridization Analysis of Transgenic Rice Plants

Integration of the transferred genes into the rice genome of the first generation (R_0) transgenic rice plants was confirmed by DNA blot hybridization analysis using the *COR47* or *HVA1* coding region as the probe. Genomic DNA was isolated as described by Zhao et al. (1989). For DNA blot hybridization analysis, 10 to 15 μg of DNA from each sample was digested with restriction endonuclease *Hind*III, or a combination of *Eco*RI and *Bam*HI, separated on a 1.0% agarose gel, transferred onto a nylon membrane, and hybridized with the ^{32}P-labeled *COR47* probe, as shown in Fig. 1, or the ^{32}P-labeled *HVA1* probe as shown in Fig. 2. There is a single *Hind*III site on the plasmid, thus digestion of genomic DNA with *Hind*III releases the fusion fragment containing the nucleic acid sequence of the gene of interest and the rice genomic sequence. Digestion with *Eco*RI and *Bam*HI releases the 1.0-kb fragment containing the *COR47* and *HVA1* cDNA.

Immunoblot Analysis of HVA1 and COR47 Protein Production in Transgenic Rice Plants

Protein extracts were prepared by grinding plant tissue in liquid nitrogen and homogenizing in extraction buffer containing 50 mM sodium phosphate (pH 7.0), 10 mM EDTA, 0.1% (V/V) Triton X-100, 0.1% (W/V) Sarkosyl, 10 mM mercaptoethanol, and 25 mg/ml phenylmethylsulfonyl fluoride. Mature seeds were cut into two halves, and the embryo-containing half-seeds were directly ground into fine powder and homogenized in the same extraction buffer. The homogenates were centrifuged at 5,000 x g for 5 min at room temperature. The supernatants were further clarified by centrifugation at 12,000 x g for 15 min at 4°C. The protein concentrations were determined based on the method of Bradford (1976) using a dye concentrate from BioRad (Hercules, CA). Proteins were separated by SDS-PAGE mini-gels, transferred electrophoretically to PVDF membrane using Mini Trans-Blot Cells (BioRad), blocked with 3% (W/V) BSA in TBS containing 0.05% (V/V) Triton X-100, incubated with rabbit anti-COR47 or anti-HVA1 antibody, and then incubated with goat anti-rabbit IgG alkaline phosphatase conjugate (BioRad). Secondary antibody was detected using 4-nitroblue-tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) supplied in an alkaline phosphatase immunoassay

kit from BioRad. Immunoreaction signals on the blot filters were scanned using a densitometer (Helena Laboratories, Beaumont, TX) to quantify the relative amounts of the HVA1 protein. Partially purified COR47 or HVA1 protein was used as the standard to estimate the levels of COR47 or HVA1 protein in transgenic rice tissues.

Analysis of Growth Performance of Transgenic Plants under Drought- and Salt-Stress Conditions

Evaluation of the growth performance under drought- and salt-stress conditions was carried out using the second generation (R_1) plants. These R_1 plants represent a population that include homozygous and heterozygous transgenic plants and segregated nontransgenic plants. Seeds of either wild-type rice plants or transformation procedure-derived nontransformed (NT) plants were used as control materials. They are both referred to as nontransformed control plants throughout this specification.

Seed Germination and Seedling Growth in medium

Thirty R_1 seeds from each of three transgenic rice lines and two nontransformed control plants were surface-sterilized and germinated in the dark at 25°C on three kinds of agarose media: MS, MS+100 mM NaCl, and MS+200 mM mannitol. The MS medium contains only its mineral salts. Seeds were allowed to germinate in MS+100 mM NaCl or MS+200 mM mannitol for 5 days and subsequently transferred to MS medium. To test the response of young seedlings to stress conditions, seeds were germinated in MS medium for 5 days. The 5-day-old seedlings were then divided, transferred onto two layers of Whatman paper in deep petri dishes and supplied with liquid MS, MS+100 mM NaCl, and MS+200 mM mannitol, respectively. Seedlings were grown under light at 25°C and their response to the stress conditions was monitored for 5 day.

Growth and Stress Treatments of Plants in Soil

Refined and sterilized field soil supplemented with a composite fertilizer was used to grow rice plants in the greenhouse (32°C day/22°C night, with a supplemental photoperiod of 10 hours). This growth condition has been routinely used to support normal growth of several rice varieties. Seeds were germinated in MS

medium for 7 days, and the 7-day-old seedlings were transferred into soil in small pots with holes on the bottom (8 cm x 8 cm, one plant per pot). The pots were kept in flat-bottom trays containing water. The seedlings were grown for two additional weeks before they were exposed to stress treatments. At this stage, most of the 3-week-old seedlings had three leaves, and some seedlings had an emerging fourth leaf. Two stress experiments using different sets of R_1 plants from the same R_0 transgenic line were conducted. In each experiment, 10 transgenic plants and at least 10 nontransformed control plants were used for each treatment.

(i) Non-stress: The plants were supplied with water continuously from the trays. The nontreated plants were also measured for their growth when the stressed plants were measured. Under this condition, both the transgenic plants and the nontransformed control plants grew well and did not show any significant difference in their growth performance during the entire period of experiments.

(ii) Water-stress: To start drought stress, water was withheld from the trays. The gradual but rapid decrease of water content in the soil produced a drought situation. After 5 days drought stress, the plants were re-supplied with water for 2 days to allow the wilted plants to recover. Then, the second round of water stress was carried out.

(iii) Salt-stress: Short-term severe salt-stress in the soil was produced by transferring the pots into trays containing 200 mM NaCl solution for 20 days. Then, the pots were transferred back to trays containing tap water to let the plants recover for 10 days. Salt concentration in the soil was quickly reduced by flushing the soil in the pots from the top with water and changing the water in the trays for several times during the first 2 days. A second round of salt stress was imposed after 10 days of recovery by supplying the plants with 50 mM NaCl solution for 30 days.

(iv) Low Temperature Stress: The plants were subjected to a temperature of 4° C for 5 days.

Data Collection and Statistical Analysis of Growth Performance

Before starting stress treatments, each nontransformed control plant and transgenic plant was measured for its initial height, leaf number, and length. During and after stress treatments, each plant was also measured. For statistical

analysis, the mean value of the 10 tested plants in each treatment was calculated and used for comparing the transgenic plants with the nontransformed control plants.

Example 1 - Production and Molecular Analysis of Transgenic Rice Plants Containing *COR47*

The structure of the plasmid pCU1a is shown in Figure 1. The cDNA of the *COR47* gene is located downstream of the rice actin 1 gene (*Act1*) promoter. The coding region of the bacterial phosphinothricin acetyl transferase gene (*bar*) is located downstream of the cauliflower mosaic virus (CaMV) 35S promoter. Rice suspension cells, which were supported by filter papers and precultured in solid medium, were bombarded by tungsten particles coated with the plasmid DNA pCU1a. Eighteen plates of suspension cells were bombarded in these transformation experiments. Two hundred ammonium glufosinate-resistant calli were selected and transferred onto regeneration medium. Sixty-three independent lines of plants (120 plants) were regenerated and grown in the greenhouse. More than 85% of the transgenic plants are fertile, producing various numbers of seeds. The sterility of some transgenic lines appeared unrelated to the presence of the foreign genes, since similar percentages of sterile plants were obtained in parallel experiments where the suspension cells were bombarded without plasmid DNA or with several other gene constructs.

Phosphinothricin acetyl transferase encoded by the *bar* gene can detoxify phosphinothricin-based herbicides. Twenty-nine lines of plants were first tested for herbicide resistance. When painted with 0.5% commercial herbicide BASTA™, leaves of transgenic plants showed complete resistance, whereas the leaves of nontransformed plants turned yellow and died. Among 29 lines of plants that were tested for herbicide resistance, 90% of them were resistant. The same 29 lines were further analyzed by DNA blot hybridization using the *COR47* cDNA fragment as probe, and 80% of them showed the predicted hybridization band pattern.

Digestion of plasmid pCU1a or genomic DNA from transgenic rice plants releases the 1.0-kb fragment containing the *COR47* coding region. Among 29 lines analyzed, 23 of them contained the expected 1.0-kb hybridization band. The hybridization patterns of all transgenic plants are unique except the predicted 1.0-kb hybridization band, suggesting that these transgenic lines were from independent

transformation events. Results of DNA blot hybridization are generally consistent with those of herbicide resistance test. Therefore, both the selectable marker gene and the *COR47* gene on the same plasmid were efficiently co-integrated into the rice genome. The use of a plasmid containing both the selectable gene and the *COR47* gene in conjunction with the tight selection procedure contributed to the high efficiency of regenerating transgenic plants.

In addition, several R_1 transgenic rice lines were tested for tolerance to low temperature. After subjecting 60-day-old plants to a temperature of 4°C for 5 days, most leaves of non-transformed control plants wilted and turned yellow, whereas leaves from two lines of transgenic plants remained green and relatively healthy. They looked similar to control plants grown at 25°C.

Four R_1 transgenic lines (60 days old) were also tested for salt tolerance by watering the plants with 200 mM NaCl for 20 days. All of the leaves and roots of the transgenic plants showed much faster growth as compared to the control plants, which grew very little and whose leaves became pale in color. Similar results were obtained by withholding water (known as water stress, dehydration stress, or drought stress) of transgenic and control plants. Other experiments were carried out using different levels of salt and different temperatures. Similar results were obtained.

Example 2 - Production and Molecular Analysis of Transgenic Rice Plants Containing *COR47 and HVA1*

Transgenic rice plants containing *COR47* were produced as described above in Example 1. In addition the plants were transformed with the barley LEA gene, *HVA1*.

The structure of the plasmid pBY520 is shown in Figure 2. The cDNA of the barley LEA gene, *HVA1*, is located downstream of the rice actin 1 gene (*Act1*) promoter. The coding region of the bacterial phosphinothricin acetyl transferase gene (*bar*) is located downstream of the cauliflower mosaic virus (CaMV) 35S promoter. Rice suspension cells, which were supported by filter papers and precultured in solid medium, were bombarded by tungsten particles coated with the plasmid DNA pBY520. Thirty-three plates of suspension cells were bombarded in

these transformation experiments. Two hundred ammonium glufosinate-resistant calli were selected and transferred onto regeneration medium. Sixty-three independent lines of plants (120 plants) were regenerated and grown in the greenhouse. More than 85% of the transgenic plants are fertile, producing various numbers of seeds. The sterility of some transgenic lines appeared unrelated to the presence of the foreign genes, because similar percentages of sterile plants were obtained in parallel experiments where the suspension cells were bombarded without plasmid DNA or with several other gene constructs.

Phosphinothricin acetyl transferase encoded by the *bar* gene can detoxify phosphinothricin-based herbicides. Twenty-nine lines of plants were first tested for herbicide resistance. When painted with 0.5% commercial herbicide BASTA™, leaves of transgenic plants showed complete resistance, whereas the leaves of nontransformed plants turned yellow and died. Among 29 lines of plants that were tested for herbicide resistance, 90% of them were resistant. The same 29 lines were further analyzed by DNA blot hybridization using the *HVA1* cDNA fragment as probe, and 80% of them showed the predicted hybridization band pattern.

Digestion of plasmid pBY520 or genomic DNA from transgenic rice plants releases the 1.0-kb fragment containing the *HVA1* coding region. Among 29 lines analyzed, 23 of them contained the expected 1.0-kb hybridization band. The hybridization patterns of all transgenic plants are unique except the predicted 1.0-kb hybridization band, suggesting that these transgenic lines were from independent transformation events. Results of DNA blot hybridization are generally consistent with those of herbicide resistance test, therefore both the selectable marker gene and the *HVA1* gene on the same plasmid were efficiently co-integrated into the rice genome. The use of a plasmid containing both the selectable gene and the *HVA1* gene in conjunction with the tight selection procedure contributed to the high efficiency of regenerating transgenic plants.

The novelty of the present experiment is that two different agronomically useful genes were transferred into rice at the same time. Several of the resulting transgenic plants harboring both plasmids showed an enhanced tolerance to salt and dehydration stress, as compared to transgenic plants that contain only one of the plasmids.

Although preferred embodiments have been depicted and described in detail herein, it will be apparent to those skilled in the relevant art that various modifications, additions, substitutions and the like can be made without departing from the spirit of the invention and these are therefore considered to be within the scope of the invention as defined in the claims which follow.

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WHAT IS CLAIMED IS:

1. A method of producing a cereal plant cell useful for regeneration to a low temperature stress, water stress, or salt stress tolerant cereal plant, said method comprising:

transforming a cereal plant cell with a nucleic acid molecule encoding a cold regulated protein.

2. The method of claim 1 wherein said cereal plant cell is derived from rice, wheat, corn, barley, sorghum, or millet.

3. The method of claim 2 wherein the cereal plant cell is derived from rice.

4. The method of claim 1 wherein said cold regulated protein is COR47, COR6.6, LTI30, or LTI45 protein.

5. The method of claim 1 wherein said nucleic acid encoding a cold regulated protein is the COR47 gene , the COR6.6 gene, the LTI30 gene, or the LTI45 gene of *A. thaliana*.

6. The method of claim 1 further comprising:

transforming the cereal plant cell with a second nucleic acid molecule encoding a late embryogenesis abundant protein or a cold regulated protein.

7. The method of claim 6, wherein said late embryogenesis abundant protein is a group 3 late embryogenesis abundant protein.

8. The method of claim 7, wherein said nucleic acid encoding a late embryogenesis abundant protein is the *HVA1* gene of barley.

9. The method of claim 1 wherein said transforming comprises:

propelling particles at said cereal plant cell under conditions effective for the particles to penetrate the cell interior; and

introducing a plasmid comprising the nucleic acid encoding the cold regulated protein into the cell interior.

10. The method of claim 9 wherein the plasmid is associated with the particles, whereby the plasmid is carried into the cell interior together with the particles.

11. The method of claim 10 wherein the plasmid is designated pCU1a.

12. The method of claim 1 further comprising regenerating the cereal plant cell after said transforming to form a transgenic cereal plant.

13. A transgenic cereal plant produced by the method of claim 12.

14. A seed produced by the transgenic cereal plant of claim 13.

15. A method of increasing tolerance of a cereal plant to low temperature stress, water stress, or salt stress conditions, said method comprising increasing levels of a cold regulated protein in said cereal plant.

16. The method of claim 15, further comprising increasing levels of a late embryogenesis abundant protein in said cereal plant.

17. A cereal plant cell transformed with a nucleic acid encoding a cold regulated protein that confers low temperature stress, water stress, or salt stress tolerance on a cereal plant regenerated from said cereal plant cell.

18. The cereal plant cell of claim 17 wherein said cereal plant cell is derived from rice, wheat, corn, barley, sorghum, or millet.

19. The cereal plant cell of claim 18, wherein the cereal plant cell is derived from rice.

20. The cereal plant cell of claim 17 wherein the cold regulated protein is COR47, COR6.6, LTI30, or LTI45 protein.

21. The cereal plant cell of claim 17 wherein said nucleic acid encoding a cold regulated protein is the COR47 gene, the COR6.6 gene, the LTI30 gene, or the LTI45 gene of *A. thaliana*.

22. The cereal plant cell of claim 17 further transformed with a nucleic acid encoding a late embryogenesis abundant protein.

23. The cereal plant cell of claim 22, wherein said late embryogenesis abundant protein is a group 3 late embryogenesis abundant protein.

24. The cereal plant cell of claim 23, wherein said nucleic acid encoding a late embryogenesis abundant protein is the *HVA1* gene of barley.

25. The cereal plant cell of claim 17 wherein said cereal plant cell includes a nucleic acid encoding a promoter, wherein expression of said nucleic acid encoding said cold regulated protein is controlled by said promoter.

26. The cereal plant cell of claim 25 wherein said promoter is the rice actin 1 gene promoter.

27. The cereal plant cell of claim 17 wherein said cereal plant cell includes a nucleic acid encoding a selectable marker.

28. A transgenic cereal plant regenerated from the cereal plant cell of claim 17.

29. A transgenic cereal plant regenerated from the cereal plant cell of claim 22.

30. A seed produced by the transgenic cereal plant of claim 28.

31. A seed produced by the transgenic cereal plant of claim 29.

32. A transgenic cereal plant transformed with a nucleic acid encoding a cold regulated protein that confers low temperature stress, water stress, or salt stress tolerance to the plant.

33. The transgenic cereal plant of claim 32 wherein said cereal plant is rice, wheat, corn, barley, sorghum, or millet.

34. The transgenic cereal plant of claim 33 wherein said cereal plant is rice.

35. The transgenic cereal plant of claim 32 wherein the cold regulated protein is COR47, COR6.6, LTI30, or LTI45 protein.

36. The transgenic cereal plant of claim 32 wherein said nucleic acid encoding a cold regulated protein is the COR47 gene, the COR6.6 gene, the LTI30 gene, or the LTI45 gene of *A. thaliana*.

37. The transgenic cereal plant of claim 32 wherein the transgenic cereal plant is additionally transformed with an nucleic acid encoding a late embryogenesis abundant protein that confers water stress or salt stress tolerance to the plant.

38. The transgenic plant of claim 37, wherein said nucleic acid molecule encoding a late embryogenesis abundant protein is a group 3 late embryogenesis abundant protein.

39. The transgenic plant of claim 38, wherein said nucleic acid molecule encoding a late embryogenesis abundant protein is the *HVA1* gene of barley.

40. The transgenic cereal plant of claim 32 wherein said transgenic cereal plant includes a nucleic acid encoding a promoter, wherein expression of said nucleic acid encoding said cold regulated protein is controlled by said promoter.

41. The transgenic cereal plant of claim 40 wherein said promoter is the rice actin 1 gene promoter.

42. The transgenic cereal plant of claim 32 wherein said transgenic cereal plant includes a nucleic acid encoding a selectable marker.

43. A transgenic cereal plant transformed with a plasmid that confers low temperature stress, water stress, or salt stress tolerance to the cereal plant, said plasmid comprising:

a first nucleic acid encoding a cold regulated protein;

a first promoter, said promoter located 5' to said first nucleic acid and controlling expression of said first nucleic acid; and

a first termination sequence located 3' to said first nucleic acid.

44. The transgenic cereal plant of claim 43 further comprising:

a second nucleic acid encoding a selectable marker, said second nucleic acid located 3' to said first termination sequence;

a second promoter located 5' to said second nucleic acid and 3' to said first termination sequence, said second promoter controlling expression of said second nucleic acid; and

a second termination sequence located 3' to said second nucleic acid.

45. The transgenic cereal plant of claim 43 wherein said plasmid is designated pCU1a.

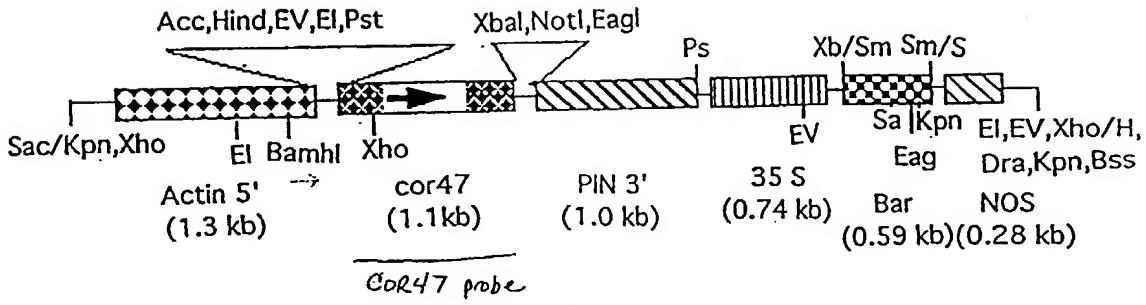
Acc,Hind,EV,EI,Pst
XbaI,NotI,EagI
Ps
Xb/Sm Sm/S
Sac/Kpn,Xho
EI
BamhI
Xho
EV
Sa Kpn
Eag
EI,EV,Xho/H,
Dra,Kpn,Bss
Actin 5'
(1.3 kb)
cor47
(1.1kb)
PIN 3'
(1.0 kb)
35 S
(0.74 kb)
Bar
(0.59 kb)
NOS
(0.28 kb)
COR47 probe

FIGURE 1

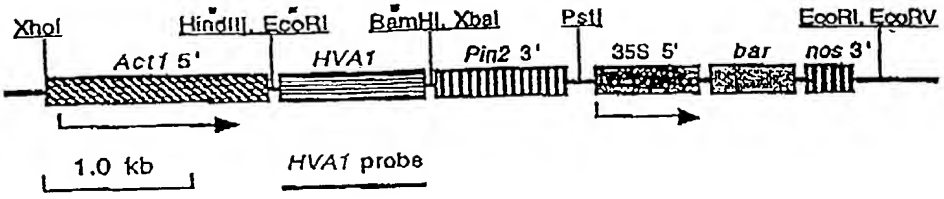
XhoI
HindIII, EcoRI
BamHI, XbaI
PstI
EcoRI, EcoRV
Act1 5'
HVA1
Pin2 3'
35S 5'
bar
nos 3'
1.0 kb
HVA1 probe

FIGURE 2